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GRANT NUMBER DAMD17-98-1-8242

TITLE: Mitosis-Specific Negative Regulation of EGF-receptor in Breast Cancer: Molecular Mechanisms, Biological Significance and Therapeutic Application

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REPORT DATE: May 1999

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | May 1999 | Annual (1 May 98 - 30 Apr 99) |

4. TITLE AND SUBTITLE
Mitosis-Specific Negative Regulation of EGF-receptor in Breast Cancer: Molecular Mechanisms, Biological Significance and Therapeutic Application

5. FUNDING NUMBERS
DAMD17-98-1-8242

6. AUTHOR(S)
Shiaw-Yih Lin

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This project is to study the M-phase specific regulation of EGF receptor (EGFR) in breast epithelial cells and to study how EGFR overexpressed cells can escape this regulation. In addition, we plan to develop a therapeutic strategy to specifically target at the EGFR overexpressors by the combination of EGF-Psuedomonas-Exotoxin (EGF-PE) with Taxol.

So far, We have observed the specific phosphorylation spots of EGFR in M phase. We are currently purifying the spots and try to identify those serine/threonine phosphorylation sites and to generate the EGFR mutant with those sites mutated. The identification and mutation of those M-phase specific sites of EGFR will be very helpful for us to understand how the activity of this receptor is cell-cycle regulated. In addition, we have completed the first step in generating the tetracycline-inducible cell line for our future studies. Finally, we have basically completed our aim 3a. We found that EGF-PE could specifically target at EGFR overexpressed cells when nocodazole or taxol was combined to enrich the M-phase population of cells. The combination of EGF-PE with the M-phase specific chemodrug, such as taxol may be a potential new therapeutic approach to treat the breast cancer patients with EGFR-overexpressing tumors in the future.

| 14. SUBJECT TERMS | | 15. NUMBER OF PAGES |
|---|---|---|
| Breast Cancer | EGFR, Mitotic Phase, desensitization, EGF-PE, taxol | 11 |
| | | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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5/24/99

PI - Signature Date

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## Introduction

This project is to study the M-phase specific regulation of EGF receptor (EGFR) in breast epithelial cells and to study how the EGFR overexpressed cells can escape this regulation. In addition, based on our previous observations, we plan to develop a therapeutic strategy to specifically target at the EGFR overexpressors both *in vitro* and *in vivo* by the combination of EGF-Psuedomonas Exotoxin (EGF-PE) with nocodazole or Taxol. If the proposed strategy works as what we hypothesized, this approach should be able to test in clinical trials to benefit breast cancer patients with EGFR-overexpressing tumors in the future.

## Body

**A. Specific aims (no change):**

1. To identify the specific phosphorylation site(s) and the kinase(s), which mediate the desensitization of EGFR in the mitotic phase of the cell cycle.
2. To determine the biological significance related to the EGFR desensitization in the mitotic phase of the cell cycle.
3. To develop a novel strategy which may specifically target at the EGFR over-expressed breast cancer cells, and to test its therapeutic potential *in vitro* and *in vivo.*

**B. Studies and Results**

In the past year, we have had some achievements in understanding the M-phase regulation of EGFR and how overexpression of this receptor can contribute to the transforming activities for the EGFR overexpressors. In addition to the studies of the molecular mechanisms of the receptor's function, we also have tested the new strategy to specifically target on those EGFR overexpressors by the combination of taxol and EGF-PE toxin proposed in the Aim III. The detail progress for the specific aims will be addressed separately as following:

Specific Aim 1:

1a: We have been performing the phosphopeptide and phosphoamino acid analysis to identify the M-phase specific phosphorylation sites on EGFR in both MDA-MB-468 and HER-5 cell lines. By comparing the profiles between the M phase, serum starved and unsynchronized cells, we have observed nine different M phase specific phosphorylated spots in either MDA-MB-468 cells or HER-5 cells. Since we are interested in a general, not cell-type specific regulation, we have been only focused on the spots appeared in both

cell lines with six in total. We are currently purifying the spots and prepare for the microsequencing with the help of the core facility available in MD Anderson Cancer Center. Once we obtain the microsequencing result, we will try to identify the putative serine/threonine phosphorylation sites by measuring the release of the labeled amino acids upon successive cycles of Edman degradation.

1b: As what we observed in EGFR, we have previously demonstrated that *neu*, another member in EGFR family, could also escape the cell cycle regulation if it was constitutively activated . Therefore, we suspected that the kinase(s), which phosphorylated and inactivated *neu* in M phase was very likely to be the same kinase inactivating EGFR. We have found that cdc2, a G2/M kinase, could physically associate with neu and phosphorylate neu *in vitro*. Therefore, we sought to determine if cdc2 could also bind to and phosphorylate EGFR. By co-immunoprecipitation, we found that cdc2 could physically associate with EGFR. We are currently constructing the GST-EGFR expression vectors. The *in vitro* phosphorylation assays will be performed to determine if EGFR can be a direct substrate for cdc2 in the near future.

Specific Aim 2:

We have started to generate the tetracycline-inducible EGFR cell lines using NR6, a EGFR null cell line as the parental cells. It usually takes two steps to generate the tetracycline-inducible lines. We have completed the first step and obtained the stable line with tet-on expression construct. When tetracycline was added, the luciferase reporter could be activated up to hundreds folds. Once we complete our aim 1, we will then be able to generate an EGFR mutant with the M-phase phosphorylation sites mutated. Then we will complete the second step in generating the tetracycline-inducible EGFR lines by transfecting the wild-type or mutant EGFR expression vectors into our tet-on contained stable cells. By comparing the transformation phenotypes between those lines, we expect to be able to determine how the escape of the negative control in M phase will allow EGFR to enhance the cellular transformation.

Specific aim 3

We have been mainly focusing on our aim 3 in the past a year or so. We have successfully produce EGF-PE toxin in our laboratory by following the protocol from our collaborator. To determine if EGF-PE could target at EGFR overexpressors in M phase, we combined EGF-PE with either nocodazole or taxol. We first tested our system by using nocodazole as an agent to synchronize cells in M phase. As shown in figure 1, we could achieve different degree of synchronization of cells in M phase dependent on the cell lines chosen. Then we treated the synchronized cells with EGF-PE and measured the cytotoxicity by MTT assay. We found that EGF-PE could preferentially kill the EGFR

overexpressed cells such as MDA-MB-468 and HER5 cells as shown in figure 1. If we normalized this effect by the percentage of synchronization, we almost achieve 100% of specific killing on the EGFR overexpressors in M phase.

Next, we used the chemotherapeutic agent, taxol instead of nocodazole to test this concept and evaluated its clinical potential. As shown in figure 2, a similar cytotoxicity as figure 1 has also been achieved. Basically we have completed our aim 3a. We are currently writing a manuscript based on this sub-aim and continuing the toxicity test *in vivo*.

## Key research accomplishments

(1) We have identified six M-phase specific phosphorylation spots of EGFR by phosphopeptide mapping.
(2) We have demonstrated that cdc2 could physically associate with EGFR *in vivo*.
(3) We have completed the first step in generating the tetracycline-inducible EGFR cell lines.
(4) We have basically completed our aim 3a. We found that EGF-PE could specifically target at EGFR overexpressed cells when nocodazole or taxol was combined to enrich the M-phase population of the treated cells.

## Reportable outcomes

Two manuscripts have been submitted with the acknowledgement of the current grant:

(1) **Lin, S.-Y.**, and Hung, M.-C. Cancer: current concepts. *Journal of Clinical Assay.*
(2) Shao, R., Hu, M., Zhou, BP., **Lin, S.-Y.**, Chiao, P., Von Lindern, R.H., Spohn, B., and Hung, M.-C. *J. biol. chem.* E1A sensitizes cells to tumor necrosis factor-induced apoptosis through inhibition of IkB kinases and NF-kB activities.

## Conclusion

We have observed the specific phosphorylation spots of EGFR in M phase. The identification and mutation of those M-phase specific sites of EGFR will help us to understand how the activity of this receptor is cell-cycle regulated and how cells can escape this negative regulation when the receptors are overexpressed. In the past, the research in this field has been mainly focused on the functions of growth factors and their receptors in G1 phase. Our studies may, in fact, open a new direction to the field for

understanding the biological and pathological functions of those receptors in the M phase of the cell cyle. Finally, the combination of EGF-PE with the M-phase specific chemodrug, such as taxol may be a potential new therapeutic approach to treat the breast cancer patients with EGFR-overexpressing tumors in the future.

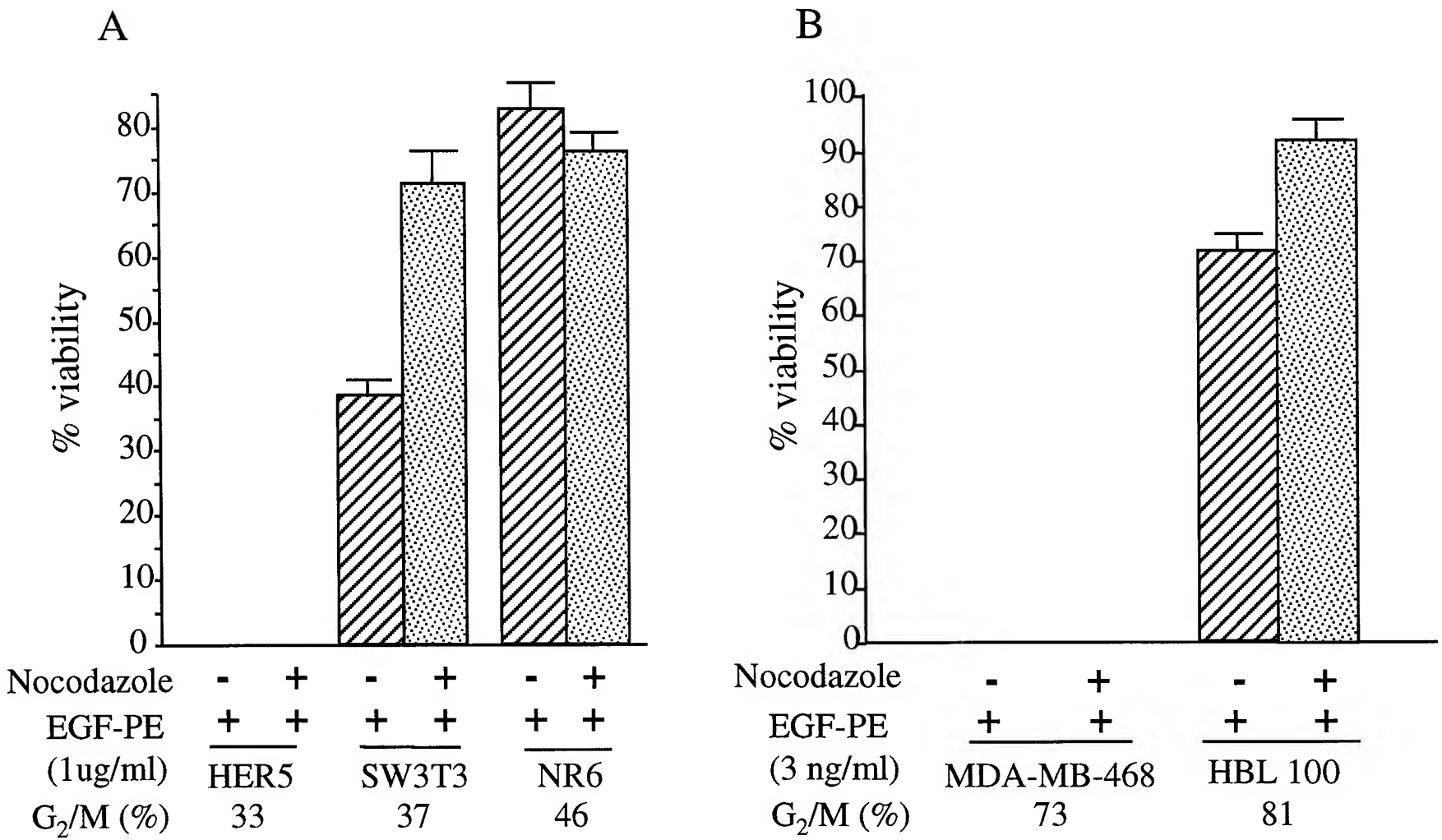


Figure1: Nocodazole protects EGFR low-expressors from killing by EGF-PE

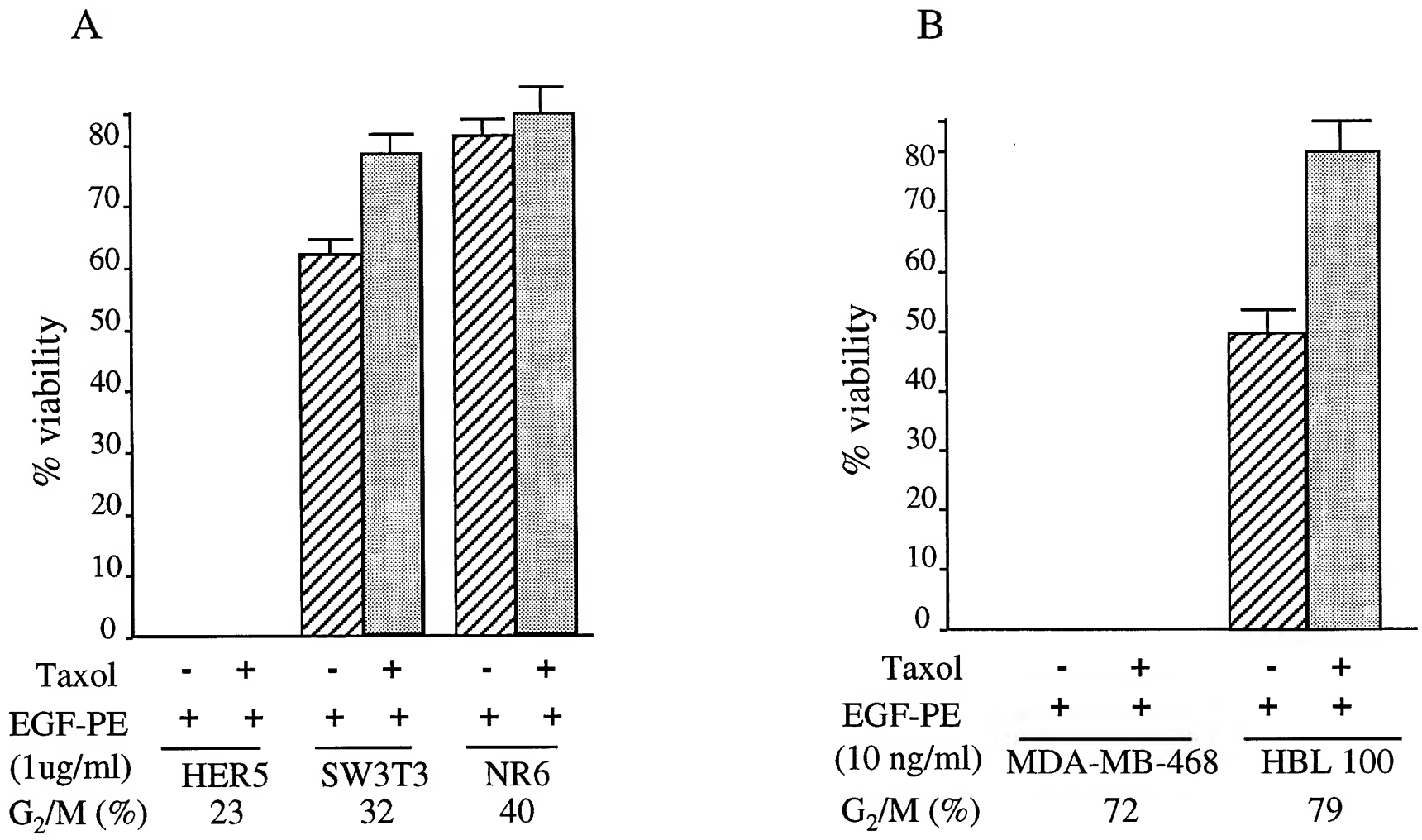


Figure 2: Taxol protects EGFR low-expressors from killng by EGF-PE

## Figure legends

Figure 1. Nocodazole protects EGFR low-expressors from killing by EGF-PE. (A) Three mouse fibroblast cell lines with the same genetic background except the EGFR expression levels (HER5: High; SW3T3: low; NR6: EGFR null) were treated with (+) or without (-) nocodazole for one day and then treated with 1ug/ml of EGF-PE. (B) The EGFR overexpressed breast cancer cell line (MDA-MB-468) and the low-expressor (HBL100) were treated as (A) except 3 ng/ml of EGF-PE was used instead.

Figure 2. Taxol protects EGFR low-expressor from killing by EGF-PE. (A) Three mouse fibroblast cell lines with the same genetic background except the EGFR expression levels (HER5: High; SW3T3: low; NR6: EGFR null) were treated with (+) or without (-) taxol for one day and then treated with 1ug/ml of EGF-PE. (B) The EGFR overexpressed breast cancer cell line (MDA-MB-468) and the low-expressor (HBL100) were treated as (A) except 10 ng/ml of EGF-PE was used instead.